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TITLE: Vasopressin Regulation and Renal Fluid and Electrolyte Handling in Rat Models of Acute and Chronic Alcohol Exposure

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**13. ABSTRACT (Maximum 200 Words)**

Fluid and electrolyte balance appears to be affected differently at different stages of alcohol use. In rat models of acute and chronic alcohol exposure and alcohol withdrawal, we systematically elucidated the role of vasopressin, an important hormone in body fluid regulation, in the physiological response to alcohol. Changes in vasopressin circulating levels do not account for altered fluid handling with alcohol exposure. Rather, alcohol-induced changes in renal responsiveness to vasopressin appear to be responsible for the pattern of diuresis, impaired water excretion, and recovery in the different phases of alcohol exposure. The primary mechanism behind this is the up and down regulation of renal vasopressin V2 receptors involved with renal tubular water reabsorption. In addition, alcohol exposure disrupts the relationships between vasopressin synthesis, brain vasopressin receptors, and blood tonicity, and thus, may affect the ability to respond to physiologic stimuli. The results of this research contribute to a better understanding of alcohol effects on vasopressin regulation of fluid handling, and should be used to implement better strategies for management of fluid and electrolyte imbalance associated with alcohol use.

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## Table of Contents

INTRODUCTION:

Alcohol use impairs renal fluid handling and the ability to maintain adequate hydration. Of importance from a military readiness aspect, is that alcohol exposure causes physiological changes in fluid and electrolyte balance that will affect soldier performance. The soldier who uses alcohol is even more susceptible to dehydration especially when water is scarce, and from a pharmacological perspective, would be more susceptible to exposure to chemical warfare agents that would reach toxic levels in the dehydrated alcohol user faster than an individual with adequate hydration.

Fluid and electrolyte balance appears to be affected differently at different stages of alcohol use. In this study, the role of vasopressin, an important hormone in body fluid regulation, in the physiological response to alcohol was examined. In rat models of acute and chronic alcohol exposure, we systematically elucidated the relationship between vasopressin synthesis in the brain, receptor regulation in the kidneys, and water and salt handling during different phases of alcohol exposure.

Our results to date have provided evidence of water imbalance with alcohol exposure that is due to altered numbers of vasopressin receptors, specifically renal V2 receptors, involved with tubular water reabsorption. Additionally, the relationships between vasopressin synthesis, brain vasopressin receptors and blood tonicity appear to be disrupted by chronic alcohol exposure.

The results of this research contribute to a better understanding of altered vasopressin regulation of fluid handling with alcohol use.

RESULTS:

We have accomplished the original goals of 1) evaluating fluid and electrolyte regulating ability in models of acute and chronic alcohol exposure and alcohol withdrawal, and 2) uncovering mechanisms behind altered fluid handling in different stages of alcohol exposure. In the process, we have developed well-characterized models of acute, chronic, and withdrawal from alcohol exposure that all exhibit similar alterations in fluid handling as found in studies of alcohol in humans. These models can be used to define mechanisms behind alcohol effects better than study of humans because conditions of alcohol dosing, hydration status, and fluid intake and output can be better controlled and monitored. We have used sensitive real-time polymerase chain reaction (qPCR) assays that we developed for quantitation of mRNA for vasopressin and vasopressin receptor syntheses to reveal the relationship between physiologic stimuli of vasopressin and vasopressin receptor synthesis. Such relationships have not been seen before with less sensitive traditional methods that could not detect fine changes in peptide levels.

In this last year, we have finished examining possible alternative explanations of impaired water handling other than renal vasopressin receptor regulation, and have determined that altered renal responsiveness to vasopressin is the main mechanism behind fluid balance perturbations seen in all phases of alcohol exposure. Also, by more closely studying stimulation of vasopressin synthesis in isolated hypothalamus-pituitary explants, as well as uncovering gender differences in renal water handling, we have identified new lines of research that need to be further explored with regards to the effect of alcohol exposure on vasopressin responsiveness to physiological stimuli.

Research accomplishments associated with each task outlined in the Statement of Work are as follows:

1. *Fluid and electrolyte regulating ability experiments*

*Animal models*: We developed and characterized dependable animal models of acute and chronic alcohol exposure and alcohol withdrawal, that exhibit normal growth and no obvious signs of organ damage or liver failure (fig. 1).

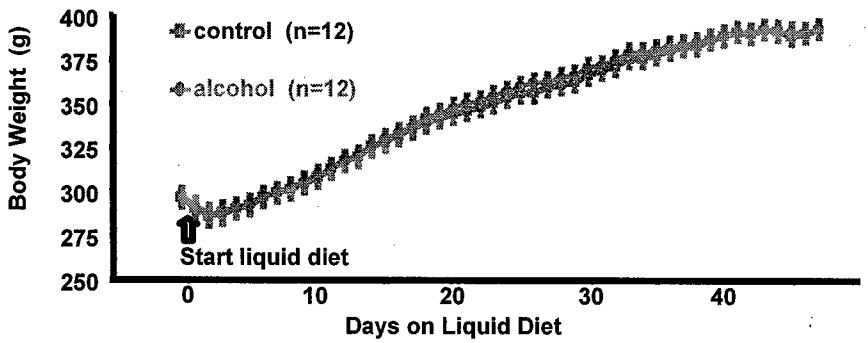


**fig. 1. Body weight of control rats and rats chronically exposed to alcohol for 8 weeks.** Rats ingest alcohol with feedings in a natural fashion that is non-stressful to the animal. Adult male Sprague Dawley rats are fed a liquid diet (80ml/day; BioServe, Frenchtown, NJ) for 8 weeks. Control group rats receive formula which does not contain alcohol. Chronic alcohol exposure group rats receive ethanol (EtOH, 3 ml/kg/day) in the formula (equal to 2-3 six-packs of beer a day in an adult human) that was adjusted to contain the same calories and nutrients as the control diet which results in similar body weight gains in both groups. (Values represent means $\pm$ s.e.m.)

These chronically catheterized animal models enable running of several experiments and obtaining multiple samples in the same animal, thus considerably reducing the total numbers of animals used. This allows for control of variability between individual animals, and generation of data with greater precision and detection of finer differences in physiologic regulation of fluid balance between groups. The repeated measures design provides for closer comparison of several arms of the experiments and simultaneous assessment of multiple aspects of fluid regulation.

The alcohol models studied thus far have been acute alcohol exposure, chronic alcohol exposure and 4 weeks of withdrawal. Because the alcohol dose chosen for these experiments was moderate (equivalent to about 2 six packs of beer a day in an adult human) the alcohol affects observed were not permanent. Our withdrawal group thus showed signs of recovery from altered fluid balance by 4 weeks of removal from alcohol and it was not necessary to study a "late phase" of alcohol withdrawal. In addition, as reversal of chronic alcohol effects occurred by simple removal of alcohol exposure, it was not necessary to examine models of "treatment" with V2 agonist or antagonist during the withdrawal phase. Findings from our V2 agonist and antagonist dose response experiments, as well as findings that there were no differences in circulating vasopressin levels in our models of chronic alcohol and alcohol withdrawal, made it no longer reasonable to treat with V2 analogues to normalize circulating vasopressin, as originally proposed. (It would still be interesting to develop more severe models of chronic alcohol exposure that would cause long-lasting effects or permanent tissue damage that was not evident in our models. We hope to pursue this different aspect of alcohol tissue injury perhaps in another future grant project.)

*Immediate effect of alcohol on vasopressin synthesis*:

We examined the immediate pharmacological effect of alcohol in contrast to the after-effects of acute alcohol once alcohol blood levels were no longer evident. Despite the traditionally accepted concept that alcohol inhibits vasopressin release and that it is the inhibition of circulating vasopressin levels that causes alcohol-induced diuresis, there are numerous reports that vasopressin levels are unchanged or even elevated after alcohol ingestion. Although circulating vasopressin levels have been measured in humans and animal models of alcohol exposure in several studies, only a few studies have ever actually documented a decrease in vasopressin circulating levels (Helderman et al, 1978; Eisenhofer and Johnson, 1982; Lepapaluoto et al, 1992), and even these have only shown just a very short term suppression at best.

While the majority of other studies have not been able to demonstrate a decrease in vasopressin levels, this has been attributed to the difficulty in detecting a suppression of already low basal vasopressin values with most vasopressin assays. Further, state of hydration of study subjects, nausea, stress due to the method and dosage of alcohol administration, or the use of anesthetized animal models, could all account for frequent reports of increased vasopressin levels after alcohol. Still, there was the possibility that perhaps vasopressin does indeed increase after alcohol as many of these reports indicate, and if so, vasopressin could not be entirely responsible for alcohol-induced diuresis.

We therefore felt it necessary to determine what was truly occurring with vasopressin levels and urine flow during elevated blood alcohol levels, especially since much of the acceptance that suppressed vasopressin levels are responsible for alcohol-induced diuresis of dilute urine is based on inference that vasopressin is the main hormone involved in renal water regulation, and indirect evidence that alcohol-induced diuresis can be prevented or reversed with

administration of exogenous vasopressin. This conclusion from indirect evidence could be faulty, however, as vasopressin administration is also able to decrease urine flow in diuresis not necessarily caused by vasopressin suppression. We thus further characterized our animal model of acute alcohol exposure, by examining the time course profiles of blood alcohol levels (fig. 2), vasopressin levels (fig. 3), and diuresis (fig. 4) during alcohol exposure.

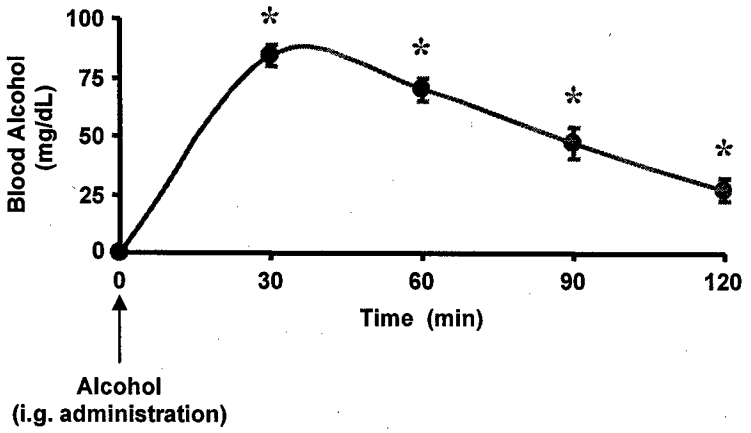


**fig. 2 Time course of blood alcohol levels.** Blood alcohol levels peak as early as 30 minutes after a single intragastric bolus of ethanol (15% v/v ethanol in water 1ml/100g body weight) and are still elevated 120 minutes after alcohol dosing. (Values represent mean ± s.e.m. n=22. * = different from time zero, $p<0.05$.)

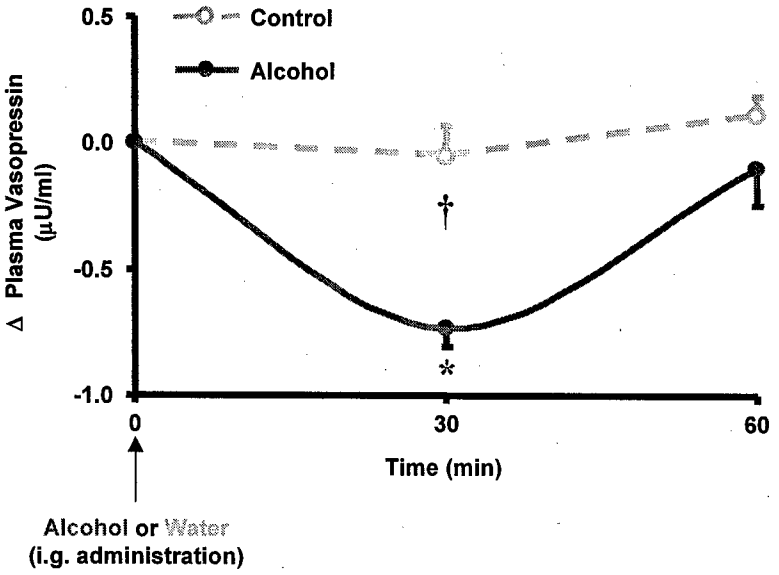


**fig. 3 Time course of circulating vasopressin levels.** A single intragastric bolus of ethanol (15% v/v ethanol in water 1ml/100g body weight; n=8) caused a transient decrease in plasma vasopressin levels at 30 minutes compared to water administration (n=7), but returned to baseline by 60 minutes. (Values represent mean ± s.e.m. * = different from time zero, $p<0.05$. + = different from control, $p<0.05$)

Blood alcohol levels immediately increased after bolus intragastric (i.g.) administration of alcohol (fig. 2), and vasopressin levels did decrease and were significantly lower than baseline 30 minutes after alcohol, but returned to baseline levels by 60 minutes (fig. 3). This was in agreement with studies that showed an immediate but transient decrease in vasopressin levels in humans (Helderman et al, 1978). This is in contrast to other studies utilizing anesthetized animal models (Cooper and Musabayane, 2000) where we suspect alcohol caused a decrease in blood pressure and renal hemodynamics resulting in increased vasopressin levels and antidiuresis. Thus our conscious animal model which enables better study of renal function without the confounding effects of anesthesia or surgical stress, we believe, reflects the true effect of alcohol on vasopressin release and renal action. Also, examining the time course of changes in blood alcohol, circulating vasopressin, and urine flow after a single i.g. bolus administration of alcohol allowed observation of the relationships between these changes better than a continuous intravenous infusion of alcohol that can also cause changes in blood pressure and hemodynamics.

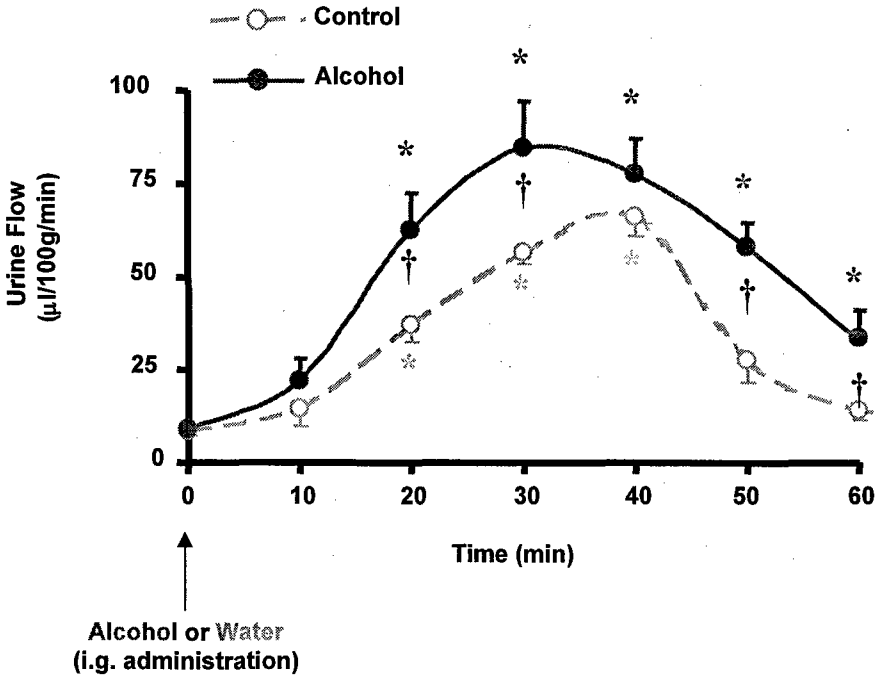


**fig. 4 Time course of diuresis.** A single intragastric bolus of ethanol (n=6) caused an increased diuresis compared to water administration in control group (n=6). Time course of increased urine flow was similar to that seen with vasopressin levels with urine flow peaking at 30 minutes and returning toward baseline by 60 minutes. (Values represent mean ± s.e.m. * = different from time zero, $p<0.05$. + = different from control, $p<0.05$)

Thus, the time course of diuresis indicates that while acute alcohol exposure may disrupt VP release momentarily, there is no prolonged deficiency of VP associated with blood alcohol levels. Rather, a brief decrease in circulating VP after alcohol intake causes an immediate diuresis. Prolonged diuresis associated with renal V2 receptor down regulation as we have

previously shown, is likely responsible for elevated VP levels reported as an after effect of alcohol. The mechanism of the temporary reduction of vasopressin levels as well as the quick return to baseline levels is unknown and warrants further study. Vasopressin synthesis does not appear to be directly pharmacologically suppressed by alcohol as normal vasopressin levels resume despite continued elevation of blood alcohol levels

*Examination of the ability of the kidneys to excrete a water load:*

Experiments testing the ability to excrete a water load for all three models of acute alcohol exposure, chronic alcohol exposure, and alcohol withdrawal showed: 1) acute alcohol exposure increased water diuresis beyond 18 hours after the last alcohol intake, even after blood alcohol levels are undetectable; 2) an impaired ability in rats with chronic alcohol exposure to excrete a water load; and 3) a reversal of the impaired water load excretion ability 4 weeks after cessation of alcohol.

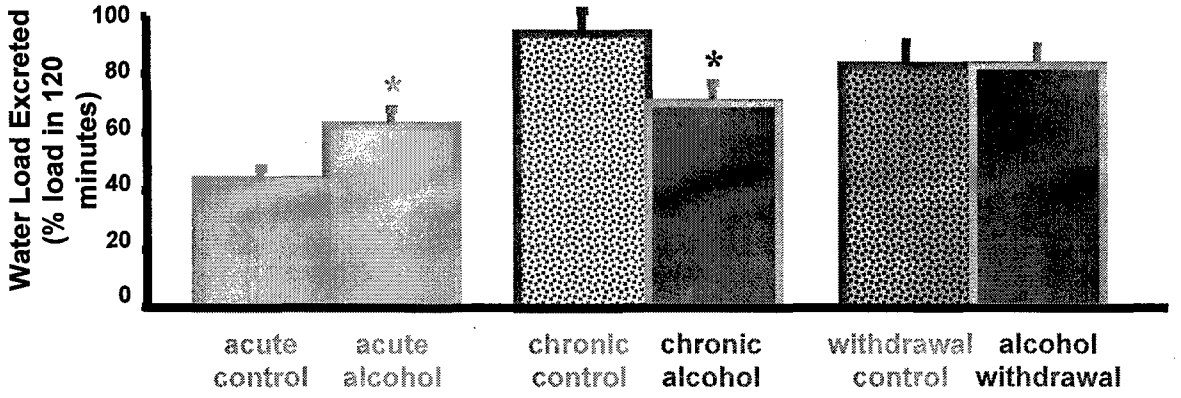


**figure 5. Water load excretion ability**. The percent of water load excreted was increased in rats 18 hours after acute alcohol intake (n=6) when blood alcohol levels were undetectable, compared to control rats (n=7). In contrast, in chronic alcohol exposure (n=9), water excretion was impaired compared to controls (n=11). Water load excretion ability returns to control levels and the percent of water load excreted was similar between rats in the alcohol withdrawal group (n=9) and control rats (n=9). (Values represent means $\pm$ s.e.m. * = significantly different from respective control, Student's t-test, $p<0.05$)

In all three phases of alcohol exposure, a difference in vasopressin secretion does not appear to be responsible for effects on water excretion, as circulating vasopressin levels were not different in any of these phases. We found that the mechanism behind altered renal water handling ability is a difference in renal responsiveness to vasopressin. We determined that regulation of renal vasopressin V2 receptor gene expression, as demonstrated by changes in vasopressin V2 receptor mRNA in the inner medulla, and renal V2 receptor binding (see results shown in section 2 below), rather than altered circulating levels of vasopressin, is responsible for the differential water load excretion abilities at different phases of alcohol exposure.

In this last year, we have examined whether there were any gender differences in water handling responses between male and female rats, as this could potentially translate into a gender difference in alcohol influence on the renal action of vasopressin. The antidiuretic responsiveness to exogenous vasopressin has been reported to be affected by gender and estrous cycle phase (Wang *et al*, 1997), but the impact of these differences on water handling is unclear. If alcohol affects water handling via altered renal responsiveness to vasopressin, we rationalized that it may be likely that alcohol effects on water handling in females may be different at different phases of the estrous cycle.

In control rats, we found that despite similar basal plasma osmolality and vasopressin levels, water load excretion progressively increased from anestrus to metestrus, and was significantly greater in metestrus compared to males (fig. 6). Metestrus has been shown to be associated with a decreased blood volume (Slimmer and Blair, 1996) despite a reduced hematocrit (Hct). Thus, the relative diuresis during metestrus that we observed possibly contributes to this decreased blood volume. This is consistent with the diuretic (via inhibition of VP stimulation of cAMP) effects of gonadal steroid hormones (Wang *et al*, 1995). This variation of water excretion abilities in the different phases of the estrous cycle indicates that future studies are needed to examine gender differences in the influence of alcohol on renal excretion.

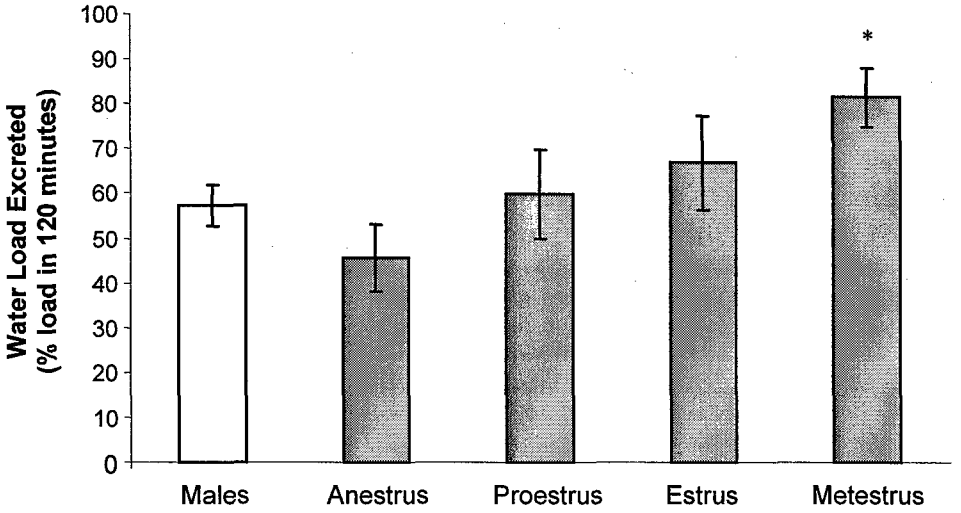


**fig. 6. Comparison of water load excretion between males and females in different phases of the estrous cycle.** Water load (2% body weight, i.g.) excretion in conscious euhydrated male (n=17) and female (F, n=13) rats were compared. Water load excretion ability varied with the different phases of the estrous cycle. (Values = mean + s.e.m.; * = dif from males, $p<0.05$)

*V2 antagonist dose response curve generation to examine the renal response to endogenous vasopressin:*

We postulated that alcohol might alter renal handling of fluid in acute and chronic alcohol use by affecting regulation of renal V2 receptors involved with tubular water reabsorption. To examine the whole animal effects of putative V2 receptor up or down regulation, we conducted V2 receptor antagonist dose response experiments in acute alcohol exposure, chronic alcohol exposure, and alcohol withdrawal models. With acute alcohol exposure, an alteration in V2 antagonist effect could not be demonstrated. This was likely

because with acute alcohol exposure the kidneys may have not yet adapted with a long-lasting change in renal sensitivity to acute changes in endogenous vasopressin levels.

In contrast, in accordance with their impaired ability to excrete a water load, rats chronically exposed to alcohol showed a blunted diuresis and a rightward shift of the dose-response curve to V2 antagonist inhibition of endogenous vasopressin (fig. 7). The suppression of V2 antagonist efficacy in increasing urine flow was due to attenuation of free water clearance in the chronic alcohol group. This decrease in V2 antagonist efficacy occurred despite no apparent differences in plasma vasopressin levels in these rats. Such results are consistent with the hypothesis that impaired ability to excrete a water load and a SIADH-like phenomenon of water retention in chronic alcohol users are due to altered renal responsiveness to endogenous vasopressin. An up regulation of vasopressin receptors in response to long-term alcohol exposure occurs (see vasopressin mRNA data below), similar to that seen with long-term exposure to vasopressin antagonists (Caltabiano and Kinter, 1991) to compensate for the initial acute alcohol-induced diuresis effect. A greater number of receptors available to bind endogenous vasopressin, require greater amount of antagonist to compete for binding sites, and thus shifts the dose-response curve.

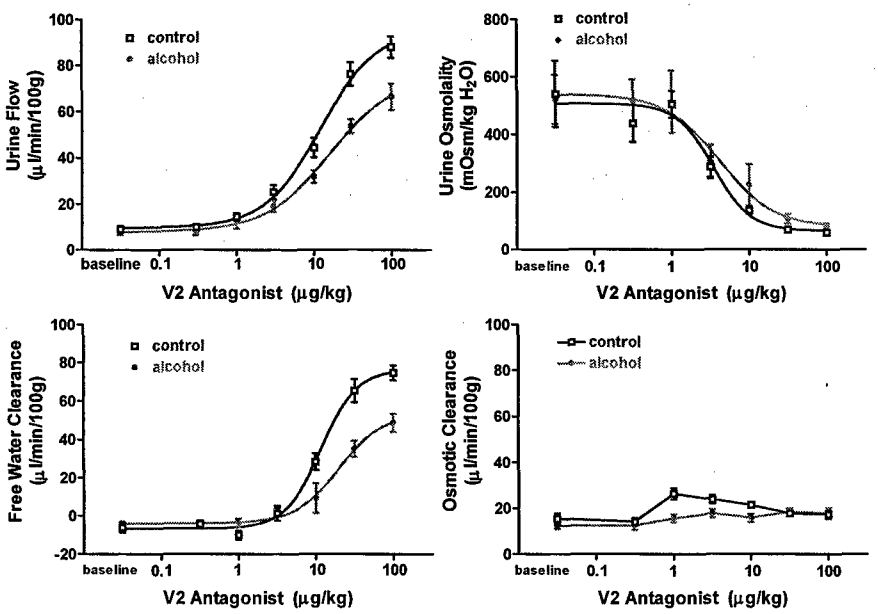


**fig. 7. Renal responses to V2 antagonist.** In accordance with their impaired ability to excrete a water load, rats chronically exposed to alcohol (n=8) showed a blunted diuresis compared to control rats (n=8) in response to V2 antagonist inhibition of endogenous vasopressin. The suppression of V2 antagonist efficacy in increasing urine flow in the chronic alcohol group was due to attenuation of free water clearance. (Values represent means ± s.e.m.)

V2 antagonist dose response experiments with the alcohol withdrawal model showed that during withdrawal from alcohol, the responses to a V2 antagonist returns to that seen in control animals. This is consistent with our findings that renal V2 receptor mRNA expression (see below), as well as the ability to excrete a water load, returns to control values during withdrawal.

Thus, a putative retention of water during withdrawal does not seem to be the result of a persistent change in renal responsiveness to vasopressin.

*V2 agonist dose response curve generation to assess maximum urine concentrating ability with maximal stimulation of vasopressin V2 receptors:*

Because altered water handling in alcohol exposed rats may be due to an alteration of the renal medullary interstitium tonicity in these animals, the urine concentrating abilities in the face of maximal vasopressin V2 receptor stimulation in these rats were examined. dDAVP dose-response experiments have verified that there is no difference in maximal urine concentrating ability between the rats chronically exposed to alcohol and control rats. Thus, these results show that there is no difference in the concentration gradient for fluid reabsorption and that altered fluid handling observed after chronic alcohol exposure is primarily due to differences in V2 receptor density. However, in rats experiencing alcohol withdrawal, the maximum urine concentration ability in response to maximal V2 stimulation with dDAVP was lower than in control rats (fig. 8). This suggests that during this phase of withdrawal, renal medullary tonicity is altered whereby reduced renal concentrating ability may help reduce water retention and return impaired water excretion to normal. The mechanism of this effect needs to be further investigated in future studies.

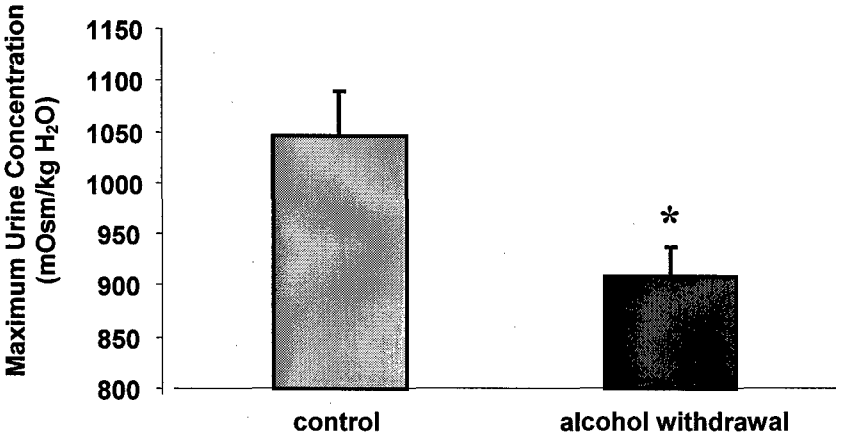


**fig. 8. Maximum urine concentration ability in rats during alcohol withdrawal.** The ability to concentrate urine in rats undergoing alcohol withdrawal (n=8) was reduced compared with controls (n=8). (Values represent means $\pm$ s.e.m. * Different from controls, $p<0.05$)

*Examination of the stimulation of vasopressin release in response to a salt load:*

Water handling impairment reported in chronic alcoholics and during alcohol withdrawal often results in a dangerous state of hyponatremia and subsequent brain seizures. We found that during alcohol withdrawal, renal vasopressin receptors and renal responsiveness to vasopressin return to normal levels. However, if water handling impairment is not due to persistent altered renal responsiveness to vasopressin, an alteration in vasopressin secretion may be implicated. Indeed, a putative rebound secretion of vasopressin after chronic alcohol exposure is believed to be involved, despite no clear evidence of elevated vasopressin levels independent of dehydration or nausea in human subjects undergoing alcohol withdrawal.

Hence, we investigated whether altered vasopressin secretion may be evident during alcohol withdrawal. We hypothesized that even if baseline circulating vasopressin levels were not different, the vasopressin response to normal physiological stimuli may be altered during alcohol withdrawal. Thus, we studied whether vasopressin release in response to a salt load may be altered during alcohol withdrawal.

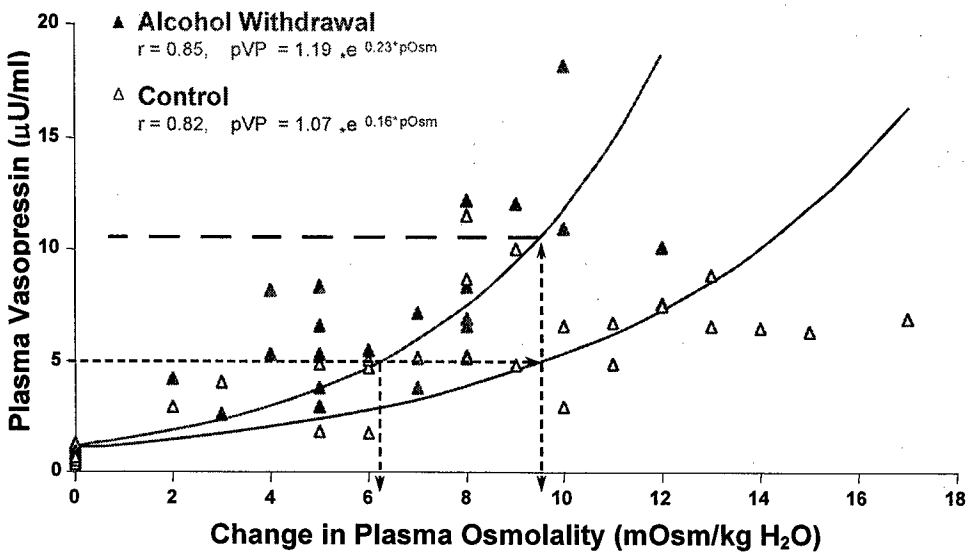


**fig. 9. Plasma vasopressin response to a given change in plasma osmolality in rats undergoing alcohol withdrawal compared to that of control rats.** Secretion of VP in response to an osmotic stimulus between rats after 4 weeks of alcohol withdrawal (n=8) and rats never exposed to alcohol (control, n=8) were compared. After basal circulating VP (pVP) and plasma osmolality (pOsm) values were obtained from conscious resting rats, blood was sampled at 20, 40, and 60 minutes during a hypertonic saline ramp (5% NaCl i.v. at 10 µl/100g/min). Samples were used to generate pOsm-pVP curves. The change in osmolality from each individual rat's starting baseline was calculated to determine the change in osmolality associated with a given vasopressin level. (Curves generated from 8 rats in each group.)

Results indicate that the relationship between baseline circulating vasopressin levels and plasma osmolality during withdrawal from alcohol is altered. The curve generated in the withdrawal group was shifted to the left of controls (fig. 9), indicating a hypersensitivity of vasopressin release during withdrawal. For example, a given level of vasopressin was triggered by a lower change in osmolality in the withdrawal group than in the control group. Likewise, the same osmotic threshold produced greater increases in vasopressin levels in the alcohol withdrawal group compared to controls. This increased stimulation of VP secretion during alcohol withdrawal is consistent with the idea of rebound VP secretion creating a condition similar to a syndrome of inappropriate antidiuretic hormone secretion (SIADH) that has been proposed to explain water retention problems reported in chronic alcoholics and during alcohol withdrawal (Trabert *et al*, 1992).

Interestingly there was no difference in the dose response curves of rats chronically exposed to alcohol and their control counterparts (fig. 10), and enhanced vasopressin secretion in

response to osmotic stimulation was not evident in rats chronically exposed to alcohol. This indicates that the impaired water excretion ability during chronic alcohol exposure is not due to hypersensitivity of vasopressin secretion or release, but rather, is mainly accounted for by altered renal responsiveness to vasopressin, as we have shown.

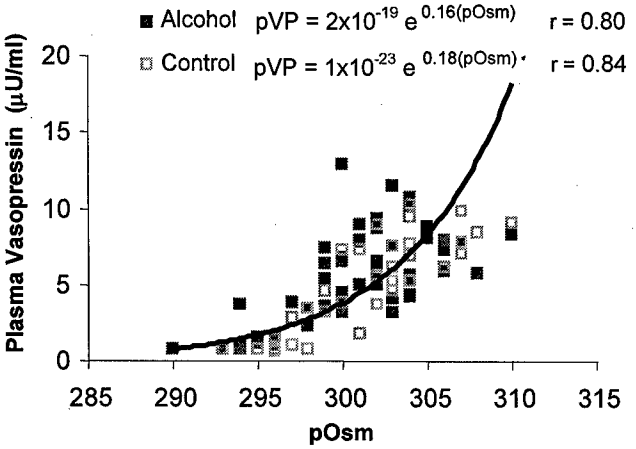


**fig. 10. Relationship between plasma osmolality and plasma vasopressin levels in chronic alcohol exposure.** The pOsm-pVP curve of animals chronically exposed to alcohol (n=12) was similar to that of control animals (n=8). The osmotic thresholds for stimulation of plasma vasopressin above 2 μU/ml were similar in both groups.

*Assessment of alcohol influence on vasopressin clearance:*

Although others have proposed that a syndrome of inappropriate vasopressin secretion may contribute to water retention seen with chronic alcohol intake, plasma vasopressin levels have not been shown to be consistently elevated. We thus examined whether higher vasopressin clearance perhaps masks enhanced basal vasopressin secretion that may occur with chronic alcohol intake. We hypothesized that because vasopressin metabolism by the kidney may be altered, and even if there was a change in brain vasopressin mRNA expression and vasopressin synthesis, circulating levels remain unchanged because vasopressin clearance also changes accordingly. If renal vasopressin receptors are affected by chronic alcohol exposure, it was possible that clearance of vasopressin from the circulation would also be affected, as it has been suggested that vasopressin renal clearance is receptor mediated (Keeler et al., 1991).

There was no difference in vasopressin clearance in either chronic alcohol (fig. 11) or alcohol withdrawal models compared to their respective controls. Vasopressin clearance, however, did increase with increasing levels of vasopressin infusion in accordance with well documented findings that vasopressin clearance and metabolism increases as a function of hormone concentration (Moses and Steciak, 1986); Uyehara and Claybaugh, 1988; Sondeen and Claybaugh, 1989). This would explain why no difference in basal plasma vasopressin levels may be evident in any of the alcohol exposure models, if basal circulating concentrations are maintained at a constant level by appropriately matched increases in vasopressin clearance.

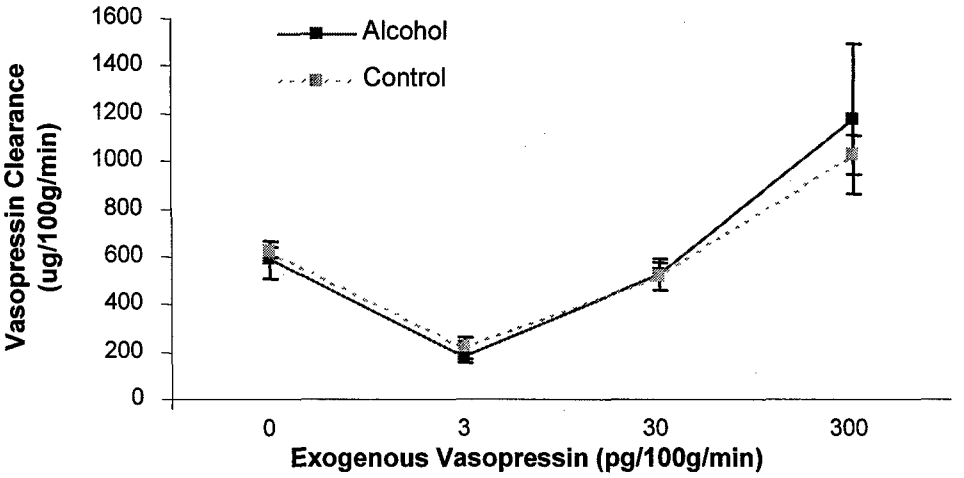


**fig. 11. Vasopressin clearance as a function of exogenous vasopressin infusion.** Vasopressin clearance at different levels of vasopressin infusion in animals chronically exposed to alcohol (n=11) was similar to that of controls (n=6). (Values represent means ± s.e.m.)

2. *In vitro assessments of tissues and samples to elucidate mechanisms behind altered fluid handling*

*Measurement of vasopressin levels in the pituitary, blood, and urine*:

Chronic alchoholism associated with water retention is supposedly due to increased circulating vasopressin or no change in vasopressin levels but an increase in renal vasopressin sensitivity, impaired renal water excretion, hyponatremia, and cirrhosis of the liver. Alcohol withdrawal, especially in patients with delirium tremens (Trabert et al., 1992) is linked to an increased plasma vasopressin concentration believed to be the result of rebound secretion of vasopressin.

Mean values of baseline plasma osmolality (pOsm), plasma vasopressin (pVP), pituitary vasopressin (pit VP), VP mRNA, V1R mRNA, and V2R mRNA of control and chronic alcohol-exposed animals were compared (fig. 12). There were no statistically significant differences detected in basal levels. We felt, however that it was probably more the relationships between these variables that needed to be examined rather than a snapshot of baseline levels, and found interesting differences between alcohol exposure and control groups in the relationships between these factors.

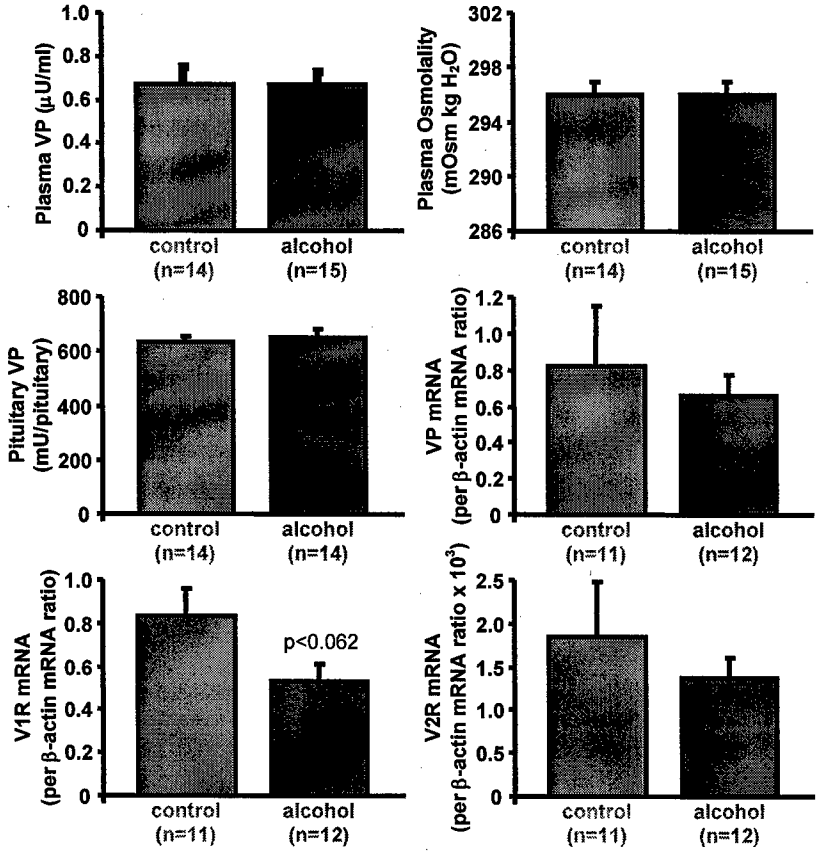


**fig. 12 Comparison of resting baseline plasma and pituitary vasopressin levels and brain mRNA gene expression.** There were no differences in plasma osmolality, plasma vasopressin, pituitary vasopressin content, brain VP mRNA, or brain V2R mRNA baseline values between control and alcohol groups. Brain V1R mRNA was not significantly different at the $p<0.05$ level, but there appeared to be a depression of the mRNA levels in most of the alcohol-exposed animals compared to controls. Values represent means $\pm$ s.e.m.

*Measurement of brain vasopressin and vasopressin receptor mRNA*

We characterized the relationships between vasopressin synthesis (VP mRNA), vasopressin release (pituitary VP, plasma VP, plasma osmolality), and vasopressin receptor regulation (V1 mRNA, V2 mRNA) in the brain, utilizing stepwise multiple regression to identify independent predictors for each of the variables. Predictor variables that showed a clear contribution to the regression equation independent of other variables were then further examined with simple linear regression, and curve fitting was done to achieve the best correlation coefficient. Stepwise multiple regression showed that the only independent predictor of VP mRNA was brain V1R mRNA. Thus, somehow, vasopressin synthesis was associated with V1 receptor up regulation. Both brain VP mRNA (fig. 13a) and plasma osmolality (fig. 13b) each significantly contributed independently to a relationship with V1RmRNA in control rats but not chronic alcohol-exposed rats.

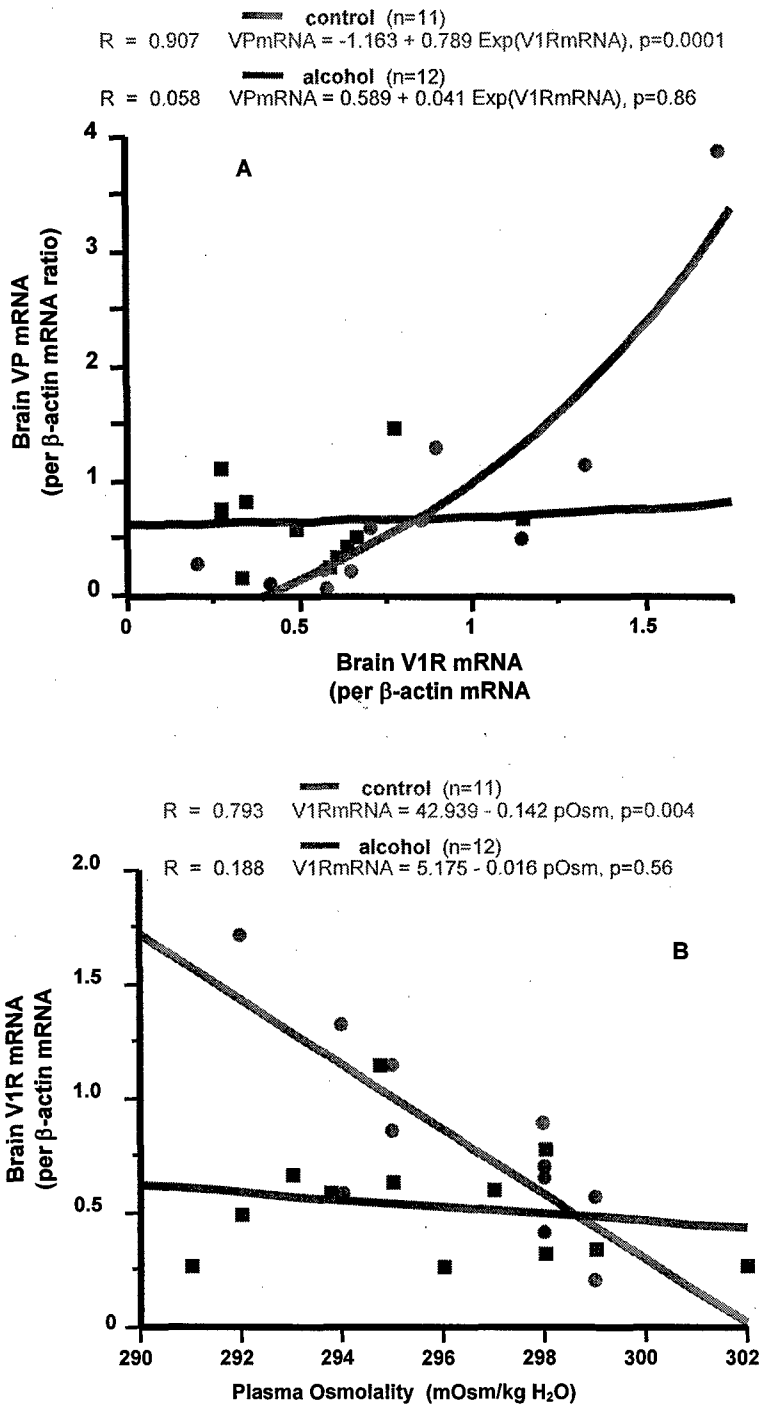


**fig. 13. Relationship between brain V1R mRNA and VP mRNA and pOsm.** Both brain VP mRNA (fig. 13a) and plasma osmolality (fig. 13b) each significantly contributed independently to a relationship with V1R mRNA in control rats. These relationships, evident in the normal physiological variability of steady state baseline values in control rats, did not exist in alcohol-exposed rats

The strong correlations between V1RmRNA, VP mRNA, and plasma osmolality, do not reveal the causal relationships between these variables. These results do indicate that brain V1R generation is highly sensitive to slight changes in plasma osmolality, and that the V1 receptor may serve as an osmotic sensor involved in the stimulation of vasopressin synthesis. Stepwise multiple regression screening of independent predictors seems to suggest a scenario where V1 receptors may participate in a positive feedback mechanism which helps amplify a vasopressin response. Thus, some stimulus causes VP mRNA to be up regulated, which induces vasopressin synthesis and an increase in circulating vasopressin. This results in action on the kidney and a resultant decrease in plasma osmolality, which causes up regulation of V1 receptors, which in turn continues to stimulate VP mRNA and vasopressin synthesis.

Regardless of the actual roles V1 or V2 receptors play in the mediation of vasopressin synthesis, it is clear from this study that alcohol disrupts normal relationships between osmotic signals, vasopressin synthesis and release, and vasopressin receptor regulation. An uncoupling of vasopressin secretion and release into the circulation has been indicated in at least one previous study where plasma vasopressin levels and plasma osmolality were shown to be increased after alcohol exposure while hypothalamic vasopressin mRNA remained unchanged (Hoffman and Dave, 1991). An inability of vasopressin synthesis to appropriately respond to physiological signals likely contributes to the body fluid imbalances seen in alcoholism. If the role of specific vasopressin receptor subtypes in the mediation of vasopressin synthesis, as suggested by our data, can be further elucidated, pharmacological strategies may be developed to correct fluid regulation problems associated with this chronic disease.

*In vitro assessment of causal relationships in the apparent uncoupling of vasopressin synthesis and physiologic stimuli that occurs with alcohol exposure:*

We established in our lab, the ability to study an isolated explant of a hypothalamus preparation with the pituitary stalk and neurohypophysis still attached. We have used this *ex vivo* explant to help define causal relationships between vasopressin brain receptors, vasopressin synthesis, vasopressin release, and osmotic stimuli. Explants were incubated for 4 hours in isotonic media and then incubated with hypertonic media (500 mOsm) for another hour. Vasopressin released into the media was collected at timed intervals, and vasopressin assayed for assessment of vasopressin release. Hypothalamic tissue vasopressin mRNA and V1 receptor mRNA, as well as pituitary vasopressin content, were measured to evaluate possible regulation of vasopressin synthesis.

Although results are still preliminary, it appears that brain explants from all three models of alcohol exposure are able to respond with vasopressin release to an osmotic stimulus when the stimulus is great enough, just as well as explants from controls (fig. 14). While there is no statistical difference in the amount of vasopressin released in response to osmotic stimulation between control and alcohol withdrawal explants, there appears to be a tendency for a higher degree of vasopressin release with alcohol withdrawal than in corresponding controls. This would be in agreement with our *in vivo* sodium load experiment findings indicating a hypersensitivity of vasopressin release exists during alcohol withdrawal. Further study examining various degrees of stimulation and signaling mechanisms behind vasopressin synthesis and release is needed to better understand the regulation of vasopressin synthesis in different stages of alcohol exposure.

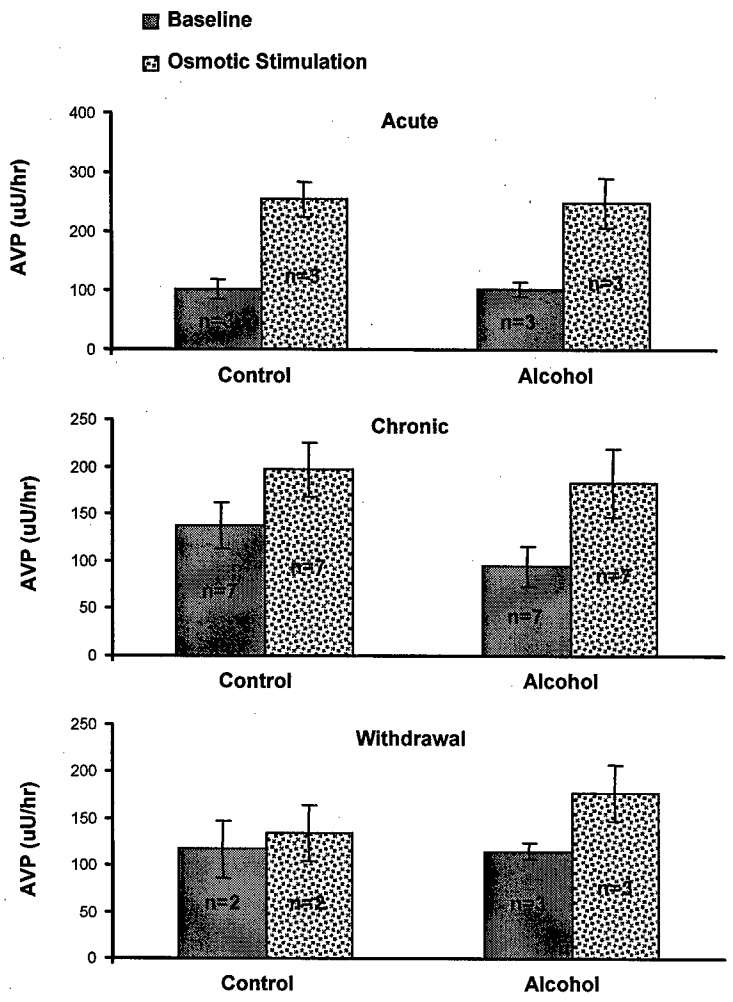


**fig. 14. Hypothalamo-Neurohypophyseal explant release of vasopressin upon osmotic stimulation.** *Ex vivo* explants of intact hypothalamo-neurohypophyseal preparations were equilibrated in isotonic media (290 mOsm) for 4 hours at which time a baseline sample was obtained. Explants were then exposed to hypertonic media (500 mOsm) for one hour before sampling for assessment of vasopressin stimulation. The hypertonic exposure stimulated vasopressin release into the media in explants from all stages of alcohol exposure. (Values represent mean $\pm$ s.e.m.)

*Examination of relationship of kidney vasopressin receptors to brain vasopressin synthesis*:

One study has suggested that stimulation of renal mechanoreceptors appear to regulate the responses of vasopressin neurons in the paraventricular nucleus of the brain (Ciriello, J., 1998). We thus felt it would be interesting to determine whether up or down regulation of receptors in the kidney may influence the regulation of vasopressin receptors or vasopressin synthesis in the brain. In examining changes in vasopressin renal receptor mRNA we found that pituitary vasopressin content was indeed related to kidney V2R mRNA in control rats (r=0.60,

p= 0.02), but not in alcohol-exposed rats (r=0.30, p=0.29) (fig. 8). It is interesting that pituitary vasopressin content appeared to be related to V2R regulation. Again, these correlations do not define a causal role V2 receptors may play in vasopressin release from pituitary stores. It is nonetheless interesting that kidney receptors may act as distant sensors to stimulate vasopressin release and decrease pituitary stores.

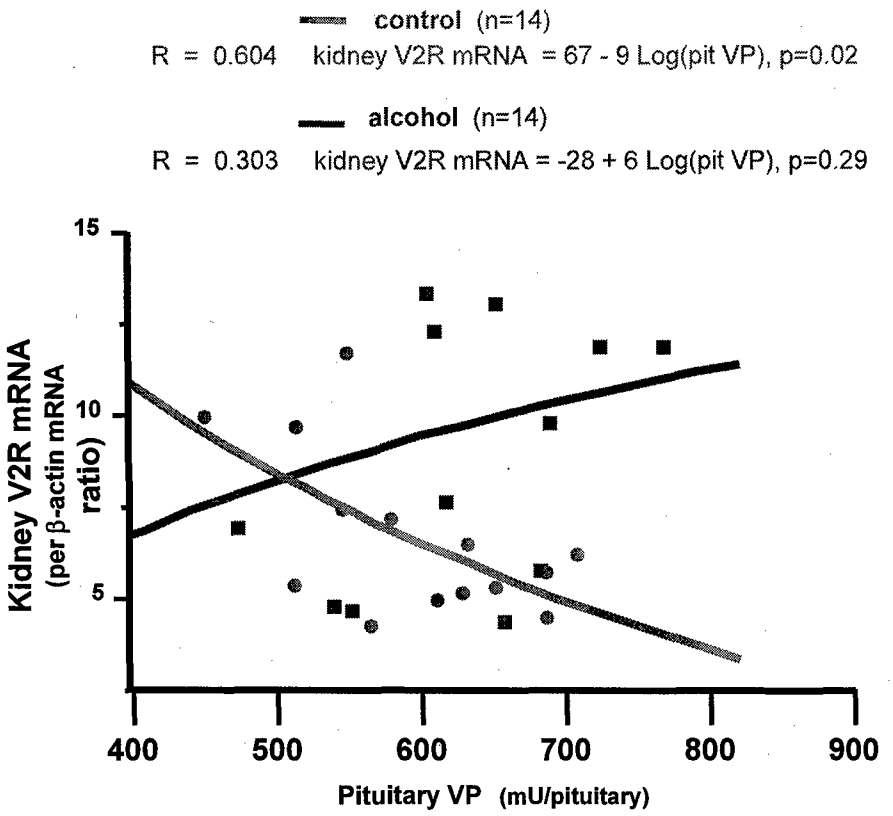


**fig. 15 Relationship between pituitary VP and V2R mRNA in the kidney.** Pituitary vasopressin content was related to kidney V2mRNA levels in control rats but not in alcohol-exposed rats.

*Measurement of kidney vasopressin receptor mRNA*:

Because our results from the whole animal experiments clearly indicated that changes in renal vasopressin receptors in the different phases of alcohol exposure was likely, we first focused our attention on assessing vasopressin V2 receptor mRNA levels in the inner medullary collecting duct. Because of the highly sensitive and reproducible measurements obtained with the real time PCR technology, we were able to detect the effects of acute alcohol, chronic alcohol, and alcohol withdrawal on renal V2 receptor synthesis that would not have been as easily detected by any other method.

As seen in figure 16, V2mRNA patterns were in accordance with water load excretion findings in all three stages of alcohol exposure. V2 receptor synthesis as indicated by the V2 to β-actin mRNA ratio was significantly less in rats acutely exposed to alcohol compared to

controls. A down regulation of renal V2 receptors is consistent with the increased water diuresis observed in the acute alcohol group. In contrast, renal V2 receptor mRNA was greater in the chronic alcohol exposure group compared to controls, which is consistent with an up regulation of V2 receptors causing the impaired ability to excrete a water load with chronic alcohol exposure. Lastly, during the withdrawal phase, V2 receptor mRNA returned toward normal as did water load excretion ability. Thus, these results indicate that the up regulation of renal V2 receptor mRNA seen with chronic alcohol exposure can be reversed upon withdrawal, similar to recently reported recovery of vasopressin mRNA in the brain seen with alcohol withdrawal (Silva et al, 2002).

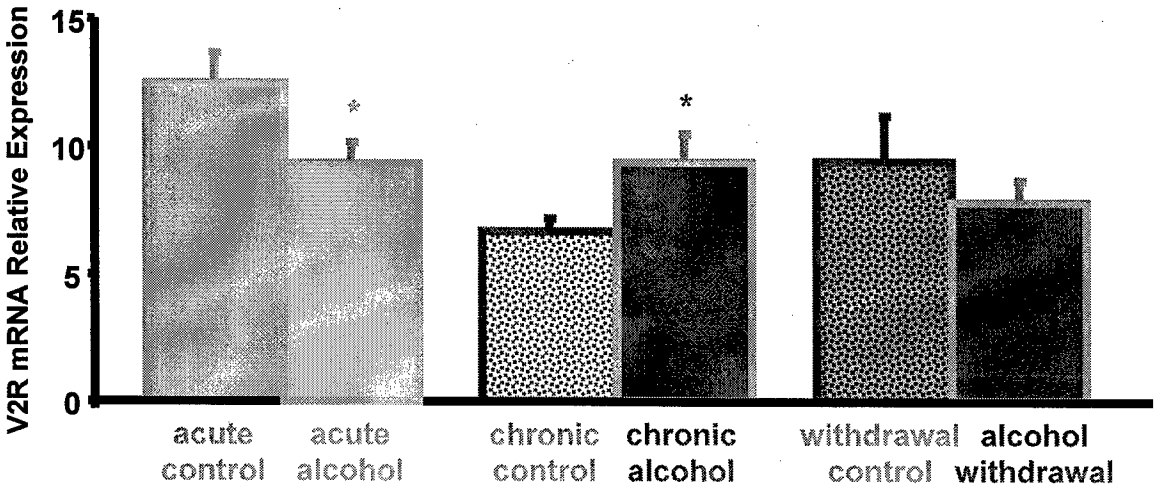


**figure 16. Renal vasopressin V2 receptor mRNA expression.** In accordance with a higher diuresis in the acute alcohol group, V2R mRNA in renal inner medulla was lower in rats treated acutely with alcohol (n=16) compared to controls (n=16). In contrast, V2 mRNA was higher in chronic alcohol rats (n=15) compared with controls (n=15). During alcohol withdrawal, there was no difference in V2 receptor mRNA between control (n=9) and withdrawal (n=9) groups. (Values represent means + s.e.m. * = significantly different from respective control, Student's t-test, $p<0.05$)

We also examined whether the up-regulation of V2 receptors was specific to the inner medulla of the kidney or whether there was non-specific up-regulation occurring throughout the kidney, or with other receptor subtypes. Thus, we compared the levels of V1 receptor (fig. 17) and V2 receptor (fig. 18) mRNA in the cortex, outer medulla, and inner medulla zones of kidneys from control (n=16) and chronic alcohol-exposed rats (n=17) using real-time PCR. Chronic alcohol-exposed caused an up-regulation of V2 receptor gene expression in the inner medulla ($8.7\pm0.9$ vs $6.8\pm0.8$ relative expression units in chronic alcohol-exposed vs control, respectively, $p<0.05$), and not in the cortex or outer medulla. For both control and chronic alcohol-exposed groups, the level of V2 mRNA was consistently highest in the inner medulla and lowest in the cortex. V1 mRNA gene expression in the control and chronic alcohol-exposed groups were similar, and there were no differences between kidney zones. Results suggest that chronic alcohol specifically targets V2 receptors. This specific up-regulation of renal V2 receptor mRNA in the inner medulla is responsible for impaired water excretion seen in chronic alcohol-exposed rats.

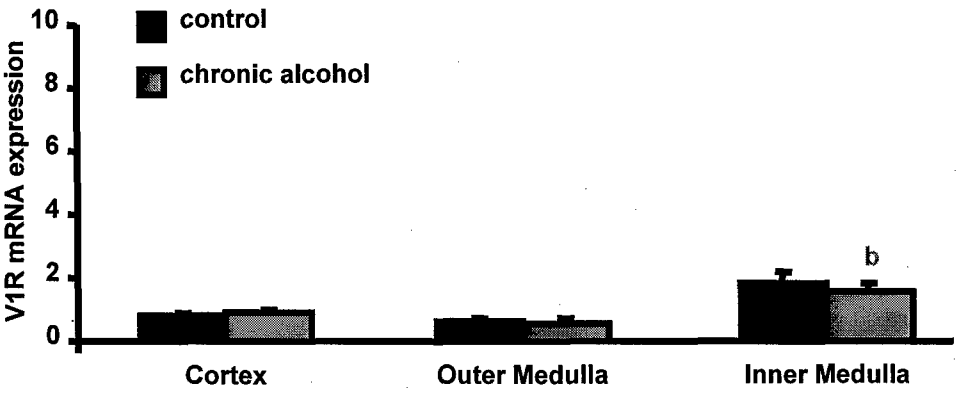


**fig. 17. V1R mRNA expression in different regions of the kidney in control rats and rats chronically exposed to alcohol.** V1R mRNA gene expression in control and chronic alcohol-exposed groups were similar. There was a tendency for V1R mRNA to be greater in the inner medulla in both control and chronic alcohol groups. (Values represent means + s.e.m. b = significant difference from outer medulla, ANOVA, $p<0.05$)

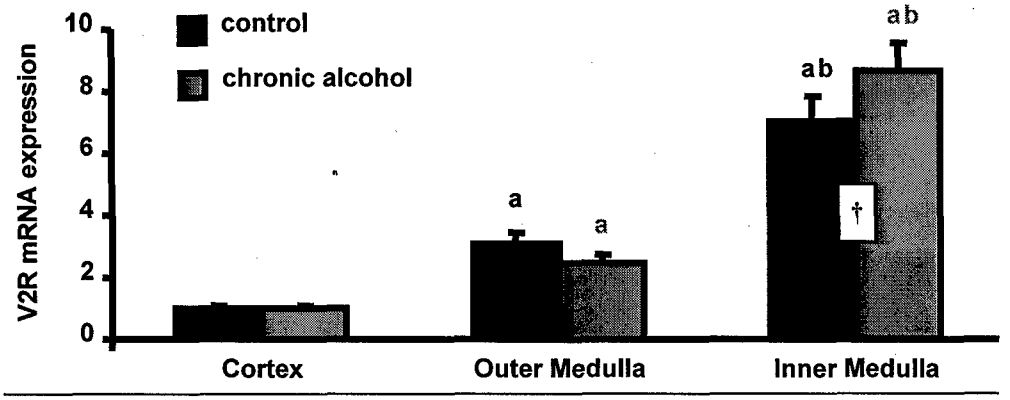


**fig. 18. V2R mRNA expression in different regions of the kidney in control rats and rats chronically exposed to alcohol.** Chronic alcohol exposure caused an up-regulation of V2 receptor gene expression in the inner medulla ($8.7\pm0.9$ vs $6.8\pm0.8$ relative expression units in chronic alcohol-exposed vs control, respectively, $p<0.05$), and not in the cortex or outer medulla. For both control and chronic alcohol-exposed groups, the level of V2 mRNA was consistently highest in the inner medulla and lowest in the cortex. (Values represent means $\pm$ s.e.m. † = significant difference between control and alcohol-exposed rats, a = significant difference from cortex, b = significant difference from outer medulla, ANOVA, $p<0.05$)

*Assessment of kidney vasopressin receptor numbers and binding affinity*:

While we initially intended on assessing vasopressin receptor numbers and binding affinity with traditional receptor binding methods, we have replaced the need to do so with the molecular methodology of the qPCR assays we developed. However, we needed to demonstrate that the mRNA assessments could be interpreted to directly translate into receptor protein synthesis and thus receptor numbers. Thus, we completed binding studies assessing receptor density in renal tissues from acute alcohol exposure and control groups. Vasopressin V2

receptor numbers and binding affinity are in accordance with the findings of the vasopressin V2 receptor mRNA measurements. V2 receptor binding in renal inner medullary collecting duct cells obtained from rats acutely exposed to alcohol is less than that of control rats, similar to the finding of reduced V2 mRNA with acute alcohol exposure.

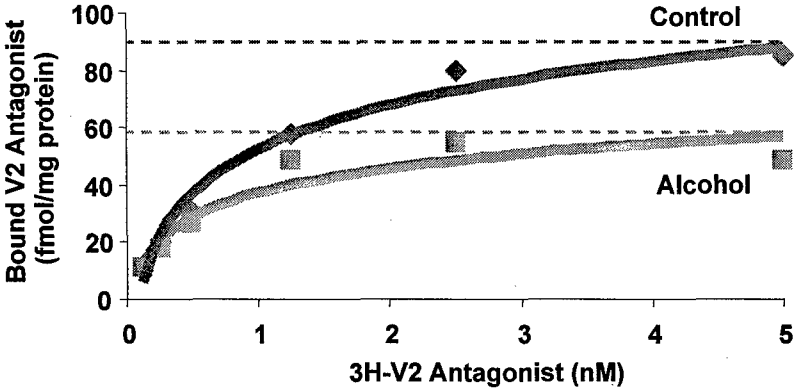


**fig. 19. V2R receptor binding analysis of kidney inner medullary collecting duct cells from rats with acute alcohol exposure and controls.** Receptor binding studies confirmed that V2 receptors in the kidney were down regulated in kidneys from rats administered alcohol acutely for 3-7 days in comparison with controls. Binding data are consistent with decreased renal V2 receptor mRNA and prolonged diuresis exhibited by acute alcohol exposure group.

*Assessment of kidney collecting duct cell function*:

So far, our quantitative assessment of V2 receptor changes are in accordance with whole animal assessment of renal water handling abilities in all phases of alcohol exposure. Thus, it was no longer critical to verify with cell physiometry methods whether changes in receptor numbers translate into changes in renal cell function.

KEY RESEARCH ACCOMPLISHMENTS:

- We demonstrated that short-term alcohol use, equivalent to 3 days of binge drinking, can alter hydration status eighteen hours after the last alcohol drink, as water diuresis persists even after blood alcohol concentrations are back to undetectable levels. This suggests that soldiers need to be adequately rehydrated after any use of alcohol to avoid fluid and electrolyte imbalances that could affect soldier performance in the field.

- We demonstrated that vasopressin levels are only transiently suppressed after acute alcohol exposure, and the persistent water diuresis seen 18 hours after acute alcohol exposure is not caused by extended suppression of circulating vasopressin. Instead, diuresis induced by acute alcohol intake is caused by a down regulation of renal V2 receptors as confirmed by V2 mRNA quantitation and receptor binding studies. This indicates that physiological changes to fluid regulation and water imbalance may persist in soldiers long after there is any evidence of alcohol intake.

- We showed that moderate chronic alcohol exposure (equivalent to about 2-3 six packs of beer a day for 8 weeks) impairs the ability of the kidneys to process water due to long term exposure to alcohol causing a compensatory up-regulation of renal receptors for vasopressin. This has implications for the effect of alcohol on the regulation of long-term body fluid balance that can lead to serious health conditions such as edema or hyponatremia.

- We determined that up regulation of renal V2 receptors in response to chronic alcohol is specific to the inner medullary zone of the kidney and specific to the V2 receptor, as up regulation was not seen with the renal V1 receptor nor in other regions of the kidney. This characterization of the specificity of the receptor involved and the localization within the target organ may help design specific drugs for the amelioration of fluid balance anomalies due to chronic alcohol intake.

- We showed that the up regulation of V2 receptors seen during moderate chronic alcohol exposure is reversible 4 weeks after termination of alcohol intake, indicating that impaired renal fluid handling can be reversed. This has implications for a recommended strategy of delaying routine field drug administration (e.g. chloroquine) for soldiers until impaired fluid handling can be reversed in order to avoid drug-induced renal toxicity that is exacerbated with alcohol.

- Results show that V1 receptors in the brain are regulated in response to changes in osmolality. This exciting finding may indicate a putative osmoreceptor role for V1 receptors in the brain or perhaps a role linking vasopressin synthesis and osmotic status. Characterization of the mechanisms behind regulation of the hormone system involved with water homeostasis may lead to a better understanding of fluid balance issues that extend beyond alcohol use effects.

- Results demonstrate that chronic alcohol intake causes the relationship between brain vasopressin synthesis, circulating vasopressin levels, V1 receptors, and plasma

osmolality, to be disrupted. This indicates the loss of appropriate linking of the blood levels of this important water regulating hormone with the message the brain receives to synthesize vasopressin in response to altered hydration status. Thus, chronic alcohol intake may have serious consequences in a soldier's ability to adjust to normal physiological stimuli such as dehydration.

- Salt loading experiments revealed rebound effects in vasopressin regulation that appear to occur in response to withdrawal from chronic alcohol exposure as evidenced by a shift in a plasma osmolality- plasma vasopressin relationship. The same physiological stimulus (change in blood saltiness) in both alcohol withdrawal and control groups caused a hypersensitive release of vasopressin into the blood during alcohol withdrawal. This likely explains the water retention often reported during alcohol withdrawal which causes increased likelihood of developing a dangerous state of hyponatremia and brain seizures. This finding could help design pharmacologocial interventions.

REPORTABLE OUTCOMES

- Publications/Presentations
  - Published abstract and presentation at Experimental Biology 2001:
    CFT Uyehara, CA Burghardt, GM Hashiro, and DA Person. After effects of acute alcohol exposure on renal water handling and responsiveness to vasopressin.
    *FASEB J.* 15(4):A134 (Abstract 154.1), 2001 and
    *J. Investigative Medicine* 49(2):249A (Abstract 328), 2001.

  - Published abstract and presentation at Experimental Biology 2002:
    CFT Uyehara, CA Burghardt, DPY Cheng, GM, Hashiro, AK Sato, and JR Claybaugh. Chronic alcohol exposure causes impaired water excretion and decreased renal efficacy of a V2 antagonist. *FASEB J.* 16(5):A837-A838, 2002.

  - Published abstracts and presentations at Experimental Biology 2003:
    Uyehara CFT, J Wu, CA Burghardt, and AJ Marean. Alcohol withdrawal reverses increased renal vasopressin sensitivity in rats chronically exposed to alcohol. *FASEB J.* 17(5): A929 (Abstract 588.15), 2003.

    Uyehara CFT, J Wu, JR Claybaugh, AK Sato, and GM Hashiro. Alcohol exposure disrupts the relationship between brain vasopressin V1 receptors and vasopressin synthesis. *FASEB J.* 17(5): A931-A932 (Abstract 588.26), 2003.

    Wu J, GM Hashiro, and CFT Uyehara. Difference in fluid handling following acute and chronic alcohol exposure in rats is due to altered regulation of renal vasopressin V2 receptor. *FASEB J.* 17(5): A928-A929 (Abstract 588.12), 2003.

    Claybaugh JR, DS Knee, AK Sato, and CFT Uyehara. Vasopressin (VP) and thirst alteration in adult rats prenatally exposed to ethanol. *FASEB J.* 17(5): A929 (Abstract 588.16), 2003.

    Knee DS, AK Sato, and CFT Uyehara and JR Claybaugh. Vasopressin (VP) and thirst alteration in adult rats prenatally exposed to ethanol. *Pediatric Res.* 53(4): 425A-426A (Abstract 2406), 2003.

  - Published abstracts and presentations at Experimental Biology 2004:
    Uyehara CFT, AJ Marean, CA Hernandez, J Wu, and GM Hashiro. Stimulation of vasopressin secretion is enhanced during alcohol withdrawal. *FASEB J.* 18(5): Abstract 6975, http://www.faseb.org/eb2004 cite/, April 2004

    Uyehara CFT, J Wu, AJ Marean, CA Hernandez, and GM Hashiro. Prolonged diuresis after alcohol ingestion is not due to persistent suppression of plasma vasopressin. *FASEB J.* 18(5): Abstract 7655, http://www.faseb.org/eb2004 cite/, April 2004

Sato AK, AK Sato, J Wu, and CFT Uyehara. Vasopressin V2 receptor gene expression in the inner medulla is specifically up-regulated with chronic alcohol exposure. *FASEB J.* 18(5): Abstract 3541, http://www.faseb.org/eb2004_cite/, April 2004

- Presentation at The Department of Defense Peer Reviewed Medical Research Program Military Health Research Forum, San Juan, Puerto Rico, 25-28 April 2004. (Hosted by The U.S. Army Medical Research and Materiel Command):

  Uyehara CFT, Wu J, Hernandez CA, Hashiro GM, Marean AJ, Claybaugh JR, Sato AK, Cheng DPY. Vasopressin regulation and renal fluid handling in rat models of acute and chronic alcohol exposure

- Submitted for presentation at the XXXV International Congress of Physiological Sciences 2005, San Diego, CA, 31 March – 5 April 2005

  Uyehara CFT, AJ Marean, J Wu, CA Hernandez, GM Hashiro, JR Claybaugh. Gender difference in water excretion is associated with estrous cycle phase and lower resting blood pressure in female rats.

  Claybaugh JR, JM Lim, AK Sato, DN Bird, DS Knee, CFT Uyehara. Temporal effects of prenatal ethanol exposure on drinking behavior, vasopressin, and oxytocin in the rat.

  Uyehara CFT, J Wu, AJ Marean, GM Hashiro, CA Hernandez. Enhanced vasopressin secretion is not evident in rats chronically exposed to alcohol.

- Animal models for acute and chronic alcohol exposure and withdrawal from alcohol for precise administration of alcohol that provide a consistent response have been developed for assessment of renal fluid and electrolyte handling. These models also make efficient use of animals enabling reduction of numbers of animals used in research.

- Molecular assays utilizing quantitative PCR technology for measuring gene expression of vasopressin and vasopressin V1 and V2 receptors have been designed and established by our laboratory. These assays allow highly sensitive detection of subtle physiological changes in vasopressin receptor mRNA and protein synthesis. These assays allow uncovering physiological signals for regulation of hormone synthesis and action at the effector organ that could not previously be done with traditional methods.

- Postdoctoral fellowship pharmacology training of one new scientist, and biomedical research training of 3 undergraduate women, were supported by this award.

- Animal model and molecular biology methods established by this project have led to development of a fetal alcohol project and award of a septic shock grant used to support Army Graduate Medical Education residents and fellows.

CONCLUSIONS:

Fluid and electrolyte balance is affected differently at different stages of alcohol use. In this study, we examined the role of vasopressin in the physiological response to alcohol in three different phases of alcohol exposure: acute alcohol exposure equivalent to binge drinking, moderate chronic exposure equivalent to about 2-3 six packs of beer a day for 8 weeks, and during alcohol withdrawal. Results suggest that alcohol-induced changes in renal responsiveness to vasopressin causes the pattern of diuresis, impaired water excretion, and recovery of water handling in the different phases of alcohol exposure. Despite evidence, reported by others, of impaired renal fluid handling, hyponatremia, and water retention in chronic alcohol exposure and during alcohol withdrawal, the renal mechanisms involved, and the role of altered vasopressin action in the kidney had not been elucidated prior to this study.

In all three phases of alcohol exposure, a difference in vasopressin secretion does not appear to be responsible for effects on water excretion, as circulating vasopressin levels were not different in any of these phases. While a transient decrease in circulating vasopressin levels occurs immediately after acute alcohol intake, vasopressin levels return to baseline levels while alcohol is still present in the blood, and there is no prolonged deficiency of vasopressin associated with the prolonged diuresis that persists after acute alcohol intake. Likewise, despite water excretion impairment during chronic alcohol intake and alcohol withdrawal, no compensatory increase in circulating vasopressin can be demonstrated during these phases. Our examination of vasopressin clearance leads us to believe this is perhaps due to any putative increases in vasopressin secretion being appropriately matched by ncreases in vasopressin clearance resulting in maintenance of circulating vasopressin at a constant level. Thus, changes in vasopressin circulating levels do not appear to account for altered fluid handling with alcohol exposure.

Rather, the main mechanism behind altered renal fluid handling with alcohol exposure appears to be altered renal sensitivity to vasopressin. We found that regulation of renal vasopressin V2 receptor gene expression, as demonstrated by changes in vasopressin V2 receptor mRNA in the inner medulla and renal V2 receptor binding, is responsible for the differential water load excretion abilities at different phases of alcohol exposure. Prolonged diuresis during the acute phase of alcohol exposure is associated with a decrease in V2 receptor mRNA and a down-regulation of V2 receptors. During chronic alcohol exposure, V2 receptor mRNA is up-regulated, resulting in an increased renal efficacy of vasopressin which causes enhanced water reabsorption and an impaired ability to excrete a water load. During alcohol withdrawal, V2 receptor mRNA returns toward normal levels, demonstrating that moderate chronic alcohol-induced impairment of renal water excretion is reversible. In addition, during alcohol withdrawal, a decrease in maximum urine concentrating ability occurs which would help compensate for fluid retention resulting from the up regulation of renal V2 receptors during chronic alcohol exposure.

Also in this project, we have for the first time systematically characterized the relationship between vasopressin gene expression in the brain, vasopressin synthesis and release and vasopressin receptor regulation during chronic alcohol exposure and withdrawal. In the process, we uncovered a relationship between brain vasopressin receptors and plasma tonicity (a discovery with potential impact on leading to a better understanding of fluid balance issues that

extend beyond that of alcohol effects). Chronic alcohol exposure disrupts normal relationships between vasopressin synthesis, vasopressin release, vasopressin receptor regulation, and blood tonicity. The important implication of this finding is that alcohol-induced changes in vasopressin regulation may affect the vasopressin response to physiologic stimuli.

Indeed, we found that the sensitivity of the vasopressin system appears to be altered during alcohol withdrawal. Osmotic stimulation studies used to create plasma osmolality-vasopressin dose response curves revealed heightened vasopressin responsiveness to changes in plasma tonicity during alcohol withdrawal. This mechanism may underlie water retention problems reported in chronic alcoholics. Further examination of relationships between brain vasopressin mRNA and pituitary VP levels indicated that a hypersensitive release of vasopressin from pituitary stores rather than a constant elevated increase in vasopressin synthesis may be the mechanism behind increased sensitivity to physiological stimuli during alcohol withdrawal.

Most recently, we have uncovered gender differences in water handling that suggests that there may be gender differences in the disruptive effects of alcohol as well, and that these gender and estrous cycle phase differences need to be further studied. Also, development in our lab of an *ex vivo* isolated brain-pituitary preparation allows us to better study causal relationships in the mechanisms behind vasopressin synthesis for future studies. Lastly, our models of alcohol exposure did not appear to cause any permanent tissue damage with the moderate levels of alcohol used, and so all physiological effects on the vasopressin and renal function systems were reversible by simply withholding alcohol intake during a washout period. It will be interesting in future studies to compare these results against effects of more severe alcohol exposure which causes tissue damage.

In summary, this project has revealed in a systematic fashion, the mechanisms behind fluid handling disturbances in different phases of alcohol exposure. Results implicate alterations in vasopressin responsiveness of the kidneys, as well as alterations in the regulation of vasopressin synthesis in the brain, being responsible for fluid handling problems with alcohol use. These findings should be used to implement better strategies for management of fluid and electrolyte imbalance associated with alcohol use. This will benefit military operational readiness by helping to provide medical countermeasures for soldiers who use alcohol.

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